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Effect of *Mycobacterium tuberculosis*-Specific 10-Kilodalton Antigen on Macrophage Release of Tumor Necrosis Factor Alpha and Nitric Oxide

Vladimir Trajkovic,[1,2] Gyanesh Singh,[1] Balwan Singh,[1] Sarman Singh,[3] and Pawan Sharma[1]*

Immunology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067,[1] and Department of Laboratory Medicine, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029,[3] India, and Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Belgrade, Yugoslavia[2]

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Secreted proteins of *Mycobacterium tuberculosis* are major targets of the specific immunity in tuberculosis and constitute promising candidates for the development of more efficient vaccines and diagnostic tests. We show here that *M. tuberculosis*-specific antigen 10 (MTSA-10, originally designated CFP-10) can bind to the surface of mouse J774 macrophage-like cells and stimulate the secretion of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α). MTSA-10 also synergized with gamma interferon (IFN-γ) for the induction of the microbicidal free radical nitric oxide (NO) in J774 cells, as well as in bone marrow-derived and peritoneal macrophages. On the other hand, pretreatment of J774 cells with MTSA-10 markedly reduced NO but not TNF-α or interleukin 10 (IL-10) release upon subsequent stimulation with lipopolysaccharide or the cell lysate of *M. tuberculosis*. The presence of IFN-γ during stimulation with *M. tuberculosis* lysate antagonized the desensitizing effect of MTSA-10 pretreatment on macrophage NO production. The activation of protein tyrosine kinases (PTK) and the serine/threonine kinases p38 MAPK and ERK was apparently required for MTSA-10 induction of TNF-α and NO release, as revealed by specific kinase inhibitors. However, only p38 MAPK activity, not PTK or ERK activity, was partly responsible for MTSA-10-mediated macrophage desensitization. The modulation of macrophage function by MTSA-10 suggests a novel mechanism for its involvement in immunopathogenesis of tuberculosis and might have implications for the prevention, diagnosis, and therapy of this disease.

Among various *Mycobacterium tuberculosis* products, proteins that are actively secreted into the culture medium are currently of particular interest. The idea that these proteins play an important role in the development of protective immune responses is based on findings that a protective T-cell response can be induced by immunization with live but not dead *M. tuberculosis* (31). Moreover, culture filtrate proteins (CFP) of *M. tuberculosis* have been shown to contain immunogenic components that elicit at least partial protective immunity (1). One of the CFP constituents and most promising candidates for the development of novel vaccines and diagnostic assays is a recently discovered 10-kDa protein, originally designated CFP-10 (5). CFP-10, also known as *M. tuberculosis*-secreted antigen 10 (MTSA-10), is one of the major antigens recognized by *M. tuberculosis*-specific human T and B cells (8, 14, 23, 24, 37), and it induces a strong delayed-type hypersensitivity response when injected intradermally into *M. tuberculosis*-infected guinea pigs (8, 12, 41). Since it is missing in *Mycobacterium bovis* BCG, MTSA-10 is an ideal candidate for diagnostic test that will discriminate between infected and BCG-vaccinated persons (3, 7, 41).

While secreted mycobacterial antigens are involved in development of protective immunity, they might be also responsible for clinical symptoms and complications of the ensuing disease in susceptible individuals. As macrophage microbicidal function in tuberculosis is suppressed, the interplay between *M. tuberculosis* and its host cell seems to be a crucial factor determining the outcome of the infection (18, 36). However, the direct influence of MTSA-10 on macrophage function has not been investigated thus far.

The production of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) and the highly reactive free radical nitric oxide (NO) by macrophages has been implicated in the development of the protective immune response leading to killing of phagocytosed *M. tuberculosis* (2, 10). We show here for the first time that MTSA-10 can bind to the macrophage surface and modulate the release of these important inflammatory mediators.

MATERIALS AND METHODS

Reagents. Dulbecco's modified Eagle medium and fetal calf serum were from Gibco BRL (Grand Island, N.Y.). The QIAexpressionist protein expression and purification kit was from Qiagen (Valencia, Calif.). *Escherichia coli* lipopolysaccharide (LPS) was from Difco (Sparks, Md.), mouse recombinant gamma interferon (IFN-γ) and Intertest-10 ELISA kit for interleukin 10 (IL-10) were from Genzyme (Cambridge, Mass.), and paired anti-TNF-α enzyme-linked immunosorbent assay (ELISA) antibodies and murine recombinant macrophage colony-stimulating factor were from R&D Systems (Minneapolis, Minn.). Griess reagent, *N*-hydroxysuccinimide–biotin, genistein, SB203580, polymyxin B sulfate, N^G-methyl-L-arginine (L-NMMA), N^G-methyl-D-arginine (D-NMMA), aminoguanidine, and an E-Toxate kit for LPS detection were all purchased from Sigma (St. Louis, Mo.). U0126 was obtained from Promega (Madison, Wis.), and streptavidin-fluorescein isothiocyanate (FITC) was from BD Pharmingen (San

* Corresponding author. Mailing address: International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi 110 067, India. Phone: 9111 6189358. Fax: 9111 6162316. E-mail: pawans@icgeb.res.in.

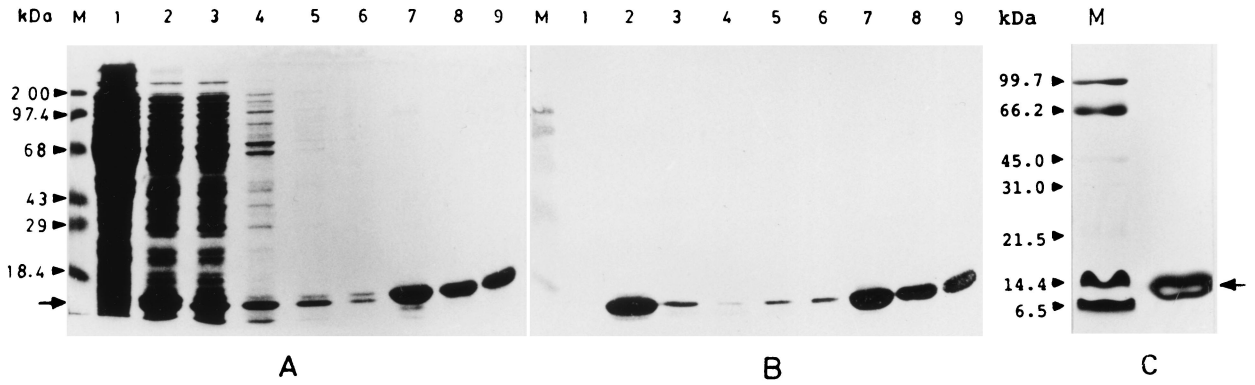

FIG. 1. Purification of the recombinant MTSA-10. The six-His-tagged MTSA-10 expressed in *E. coli* was purified by affinity chromatography using Ni-NTA agarose with the indicated imidazole concentration in the wash and elution steps. The cleared cell lysate and various chromatography fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were visualized with Coomassie blue (A) and probed with antipolyhistidine antibody after Western blotting (B). Lanes: M, molecular mass markers; 1, noninduced cell lysate; 2, IPTG (isopropyl-β-D-thiogalactopyranoside)-induced cell lysate; 3, flowthrough; 4 to 6, 20 mM imidazole washes; 7 to 9, 250 mM imidazole eluates. The arrow indicates the recombinant MTSA-10 protein band of the expected size (~10 kDa). (C) Silver staining of purified MTSA-10.

Diego, Calif.). Whole-cell lysate of *M. tuberculosis* was kindly provided by J. T. Belisle of Colorado State University (Fort Collins).

Cloning, expression, and purification of MTSA-10. The open reading frame Rv3874, encoding MTSA-10 of *M. tuberculosis*, was amplified by PCR from the genomic DNA of a local clinical isolate by using the following primers: forward, 5′-GCGGATCCCATGGCAGAGATGAAGACCG-3′; reverse, 5′-CCCAAGCTTGTCAGAAGCCATTTGCGAG-3′ (*Bam*HI and *Hin*dIII sites, respectively, are underlined). The PCR product was directly cloned in the intermediate vector pGEM-T-Easy (Promega). After its nucleotide sequence had been validated (GenBank accession no. AF419854), the full-length gene was subcloned in the bacterial expression vector pQE-31 (Qiagen) for expression as polyhistidine-tagged recombinant MTSA-10 protein in *E. coli*. Recombinant MTSA-10 was purified by nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography according to the manufacturer's recommendations for purification of proteins under native conditions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the purified protein revealed it to be an essentially homogenous preparation (Fig. 1A and C). In an immunoblot, the purified recombinant MTSA-10 was specifically recognized by a mouse antipolyhistidine monoclonal antibody (Fig. 1B). The preparation was dialyzed against several changes of phosphate-buffered saline (PBS) and stored in small aliquots at −20°C. The concentration of LPS in the MTSA-10 preparation was 0.5 ng/mg, as determined by a *Limulus* amebocyte lysate-based E-Toxate kit. For some experiments, MTSA-10 was further purified by ion-exchange high-performance liquid chromatography (HPLC), using a POROS-HQ column with the matrix consisting of cross-linked polystyrene-divinylbenzene flowthrough particles coated with fully quaternized polyethyleneimine, which is completely ionized over a pH range of 1 to 14. Buffer containing 50 mM TRIS (pH 8.4) was used as a binding buffer, while elution of bound protein was done with a 0 to 1 M gradient of NaCl in binding buffer.

Cell cultures. Mouse macrophage cell line J774.1 (American Type Culture Collection) was maintained in HEPES-buffered Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, sodium bicarbonate, glutamine, penicillin, and streptomycin (culture medium). Mouse bone marrow-derived macrophages (BMMs) were prepared from cells of the femurs of adult BALB/c mice. Briefly, femurs were flushed with culture medium, and cells were plated in complete medium containing 25 ng of murine M-CSF per ml on 10-cm tissue culture plates for 7 days in a 37°C incubator containing 5% CO_2. Mouse peritoneal macrophages were obtained from resident peritoneal cells of BALB/c mice, following 1 h of adherence to plastic at 37°C. For cytokine and NO production, cells were seeded in triplicate in flat-bottom 96-well plates (10^5 cells/well), in 200 μl of culture medium containing MTSA-10 or MTSA-10 plus IFN-γ, in the presence or absence of different kinase inhibitors. Alternatively, cells were incubated for 18 h with MTSA-10 in the presence or absence of the kinase inhibitors, extensively washed, and then stimulated with LPS or *M. tuberculosis* lysate. After 24 h of incubation at 37°C in a humidified atmosphere with 5% CO_2, cell culture supernatants were collected for determination of cytokine and NO concentrations. Mitochondrial respiration, as an indicator of cell viability, was assessed after various treatments by MTT [3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] assay, as previously described (39).

Flow-cytometric analysis. Biotinylated MTSA-10 was prepared by mixing purified MTSA-10 (1 mg/ml) in carbonate buffer with NHS-biotin (1 mg/ml in dimethyl sulfoxide) in a 1:8 ratio. Following incubation for 4 h at room temperature, the solution was dialyzed against PBS. For the fluorescence-activated cell sorting analysis, J774 cells or BMMs were washed twice with wash buffer (PBS with 1% bovine serum albumin and 0.01% sodium azide) and suspended at 10^6 cells/100 μl. Cells were incubated for 1 h on ice with biotinylated MTSA-10. After a washing, cells were incubated for 1 h on ice with streptavidin-FITC (1:2,500 in wash buffer). Following the final washing, cells were resuspended in 0.5% paraformaldehyde and analyzed on a FACSCalibur (Becton Dickinson). For the competition experiments, prior to incubation with biotinylated MTSA-10, cells were incubated for 1 h on ice with unlabeled MTSA-10.

Nitrite and cytokine determination. Nitrite accumulation, an indicator of NO production, was measured by using the Griess reagent (20). Briefly, 50-μl aliquots of culture supernatants were mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. The absorbance at 540 nm was measured in an automated microplate reader. The nitrite concentration (in micromolar units) was calculated from a $NaNO_2$ standard curve. Concentrations of TNF-α and IL-10 in cell culture supernatants were determined by ELISA using paired anti-TNF-α antibodies and an Intertest-10 ELISA kit, respectively, according to the manufacturer's instructions.

Statistical analysis. Data from representatives of at least three independent experiments with similar results are presented as means ± standard deviations of triplicate observations. The statistical significance of the difference between various treatments was analyzed by one-way analysis of variance, followed by a Student-Newman-Keuls test. A P value less than 0.01 was considered significant.

Nucleotide sequence accession number. The nucleotide sequence of the MTSA-10 gene has been assigned GenBank accession number AF419854.

RESULTS

MTSA-10 binds to the surface of J774 cells and induces TNF-α release. To assess the ability of MTSA-10 to bind to the surface of J774 macrophages, cells were incubated with biotinylated MTSA-10, and the presence of bound MTSA-10 was investigated by streptavidin-FITC staining. A significant MTSA dose-dependent shift in the fluorescence intensity was observed, indicating that the surfaces of J774 cells can bind MTSA-10 (Fig. 2A). This was further supported by competition experiments in which the increase in fluorescence was completely prevented by preincubating J774 cells with unlabeled MTSA-10 (Fig. 2A). The addition of MTSA-10 signifi-

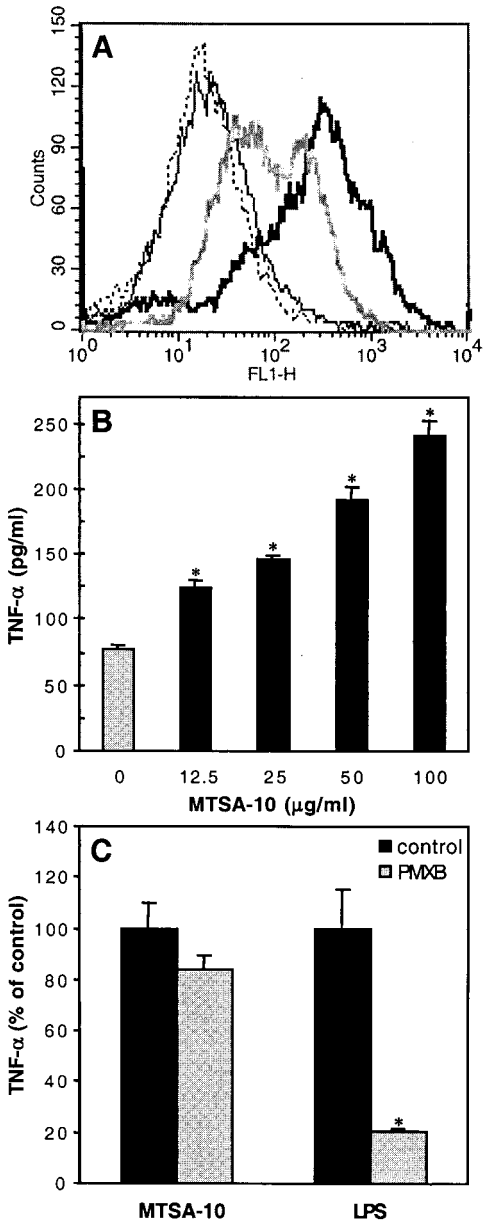

FIG. 2. MTSA-10 binds to J774 cells and induces TNF-α secretion. (A) J774 cells were incubated with biotinylated MTSA-10 (25 μg/ml [grey line] or 50 μg/ml [thick black line]) and then stained with streptavidin-FITC, as described in Material and Methods. Alternatively, before incubation with biotinylated MTSA-10 (50 μg/ml) and subsequent streptavidin-FITC staining, cells were treated with unlabeled MTSA-10 (50 μg/ml) for 1 h (thin black line). Cells stained only with streptavidin-FITC were used as a control (dotted line). (B) J774 cells were incubated with different concentrations of MTSA-10. *, P of <0.01 compared to unstimulated cultures (B) or corresponding controls (C). (C) J774 cells were incubated with 50 μg of MTSA-10 per ml or 0.1 μg of LPS per ml, in the presence or absence of 10 μg of polymyxin B (PMXB) per ml. (B and C) TNF-α accumulation was measured in cell culture supernatants after 24 h of incubation. Control values (C) were 172 pg/ml (MTSA-10) and 554 pg/ml (LPS).

cantly increased TNF-α production by J774 macrophages in a dose-dependent manner (Fig. 2B). IL-10, however, remained undetectable in the supernatant of MTSA-10-treated cultures. The effect of MTSA-10 on macrophage TNF-α release was not a consequence of LPS contamination, since it could not be mimicked by 0.05 ng of *E. coli* LPS per ml (data not shown), a concentration estimated to be present in 100 μg of our MTSA-10 preparation per ml. Furthermore, MTSA-10 caused a significant increase of macrophage TNF-α production even at 12.5 μg/ml, which contained less than 0.01 ng of LPS per ml. Finally, the LPS-inactivating agent polymyxin B did not significantly affect MTSA-10-induced TNF-α synthesis, while it totally abolished that triggered by LPS (Fig. 2C).

MTSA-10 pretreatment desensitizes J774 cells for LPS and *M. tuberculosis* lysate-induced NO release. While MTSA-10 did not affect macrophage NO synthesis when applied alone or simultaneously with the inducible-NO-synthase (iNOS) activator LPS (data not shown), pretreatment of J774 cells with MTSA for 18 h caused significant reduction of NO release upon subsequent exposure to LPS (Fig. 3A). A similar inhibitory effect of MTSA pretreatment was observed when the whole-cell lysate of *M. tuberculosis* was used to stimulate macrophage NO production instead of LPS (Fig. 3A). This was not due to a toxic or antiproliferative action of MTSA-10, as the cellular respiration assessed by the MTT assay did not differ between MTSA-10-pretreated and control cultures (data not shown). Interestingly, the effect of MTSA-10 was selective for NO, since neither TNF-α nor IL-10 release induced by LPS or *M. tuberculosis* lysate was affected in MTSA-pretreated J774 cells (Fig. 3A). LPS at 0.05 ng/ml failed to mimic, and polymyxin B did not abolish the effect of MTSA pretreatment (data not shown), confirming that macrophage desensitization was indeed mediated by MTSA-10. Moreover, in spite of comparable down-regulation of NO synthesis, macrophage reprogramming by LPS was distinct from that induced by MTSA-10, since it involved the reduction of TNF-α, as well as a significant increase of IL-10 release following the second round of LPS stimulation (Fig. 3B).

IFN-γ synergizes with MTSA-10 for NO production in J774 cells. In contrast to LPS- or *M. tuberculosis* lysate-induced NO release, MTSA-10 pretreatment failed to affect NO production in macrophages stimulated with a high dose (100 U/ml) of IFN-γ (Fig. 4A). Furthermore, although IFN-γ at lower concentration (10 U/ml) did not induce macrophage NO synthesis, it markedly potentiated *M. tuberculosis* lysate-stimulated NO production and almost completely antagonized the desensitizing effect of MTSA pretreatment (Fig. 4B). Interestingly, not only did MTSA-10 fail to suppress macrophage NO release when applied before IFN-γ stimulation, it even synergized for the induction of NO with a low dose (10 U/ml) of IFN-γ, provided that both stimuli were applied simultaneously (Fig. 4C). The synergism was lost at a lower IFN-γ concentration (1 U/ml), but the potentiating effect of MTSA-10 was preserved when macrophage NO synthesis was induced by a high dose (100 U/ml) of IFN-γ (Fig. 4D). Again, the effect of MTSA-10 could not be reproduced by 0.05 ng of LPS per ml, arguing against the involvement of the extremely low levels of contaminating LPS (data not shown). Aminoguanidine (2 mM), a selective inhibitor of iNOS, and the nonselective NOS blocker L-NMMA (500 μM) (but not its inactive counterpart, D-NMMA) both prevented the nitrite accumulation in MTSA-10–IFN-γ-stimulated cultures (12.9 ± 0.2 μM in control, versus 1.2 ± 0.4 or 0.9 ± 0.3 μM in aminoguanidine- or L-NMMA-treated cultures, respectively; $P < 0.01$), suggesting

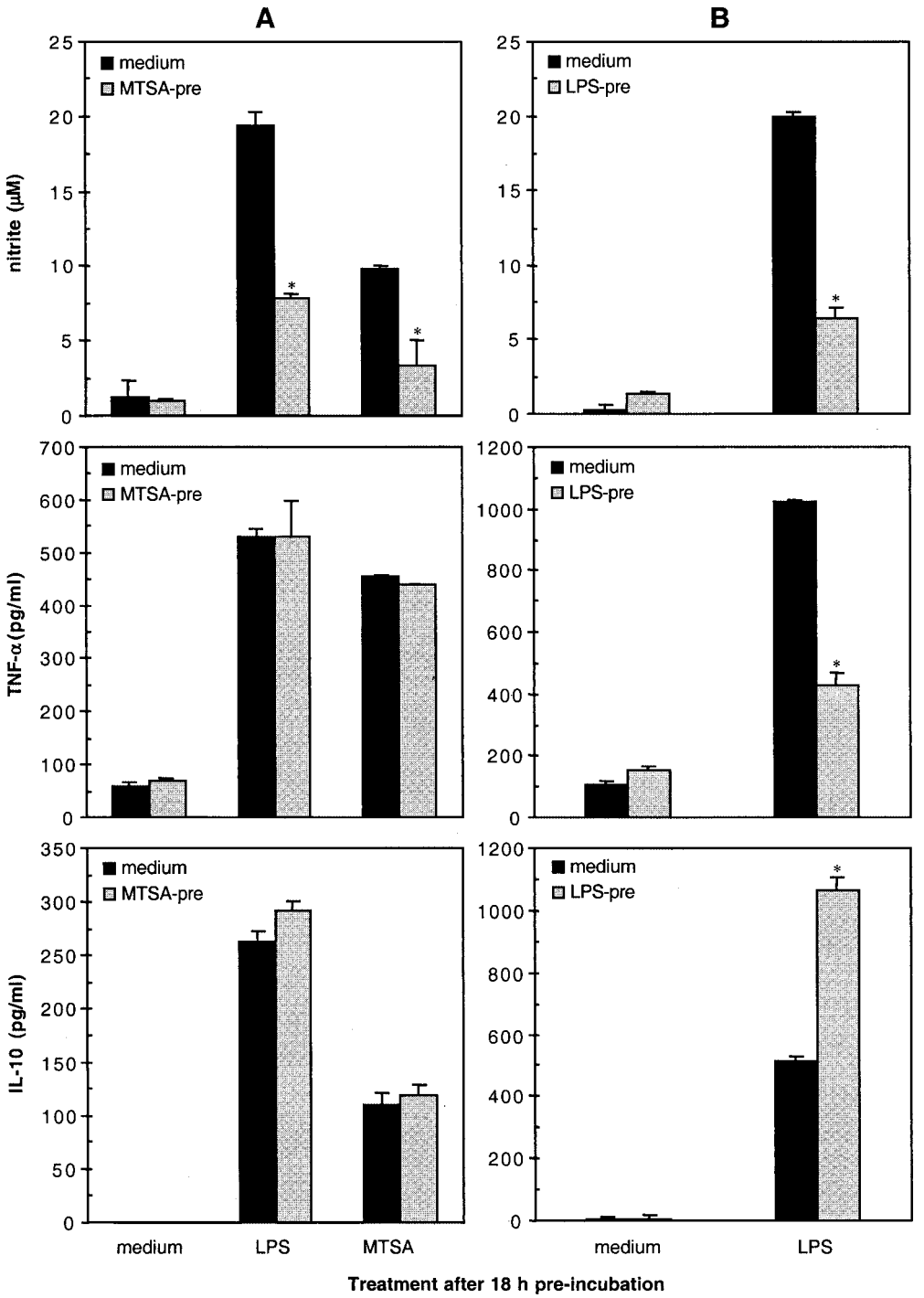

FIG. 3. MTSA-10 pretreatment desensitizes J774 macrophages for subsequent NO production. J774 cells were pretreated with 50 μg of MTSA-10 per ml (A) or 0.1 μg of LPS per ml (B) for 18 h (MTSA-pre and LPS-pre). Afterwards, cells were washed several times with culture medium, rested for 2 h, and then were stimulated with *M. tuberculosis* cell lysate (MtbLys; 50 μg/ml) (A) or LPS (1 μg/ml) (A and B). Concentrations of nitrite, TNF-α, and IL-10 were measured in cell culture supernatants after 24 h of incubation. *, P of <0.01 compared to corresponding unstimulated cultures.

that nitrite production was a consequence of a high-output L-arginine–NO pathway mediated by iNOS. The NO-inducing ability of MTSA-10 was heat stable, since it was preserved after 5 min of treatment in boiling water (data not shown). However, IFN-γ–MTSA-10-mediated induction of macrophage NO production was almost completely prevented upon MTSA-10 digestion with proteinase K (2 h at 50°C; proteinase K/MTSA-10 ratio, 1:20 [wt/wt]), thus further excluding the involvement of

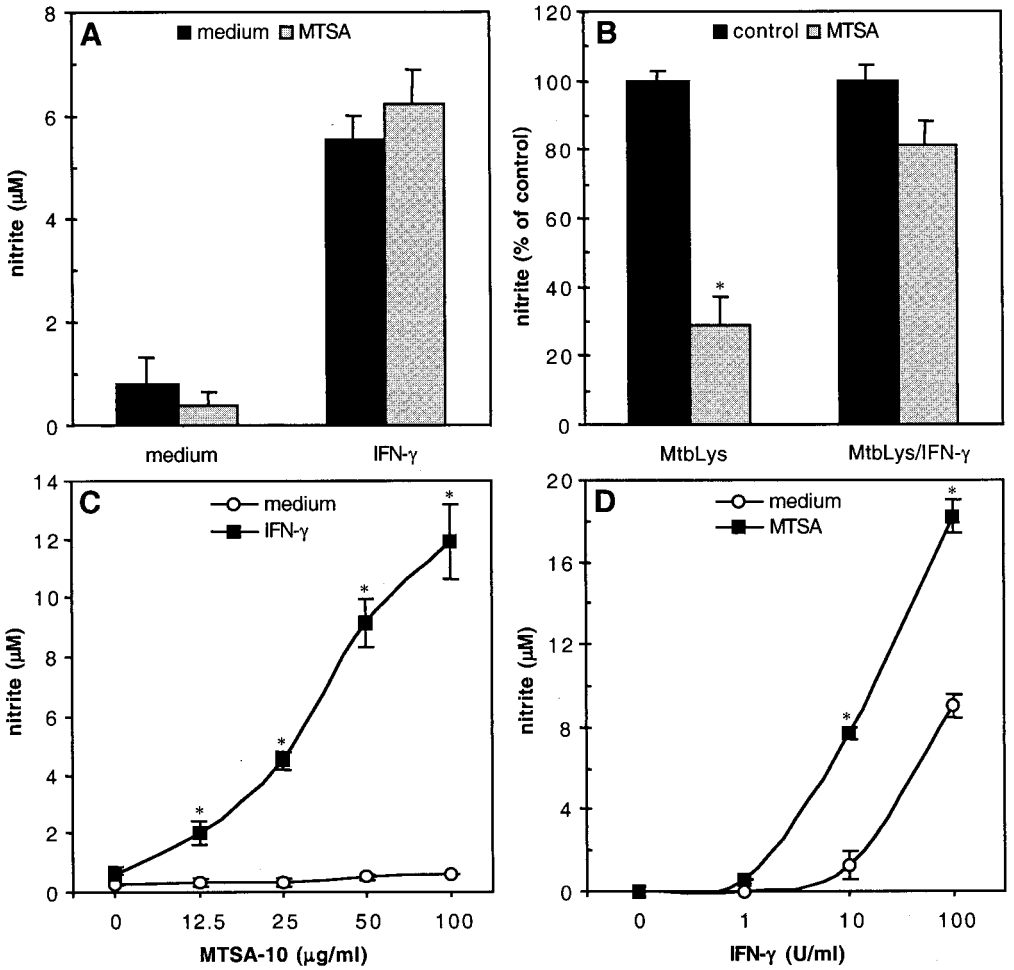

FIG. 4. MTSA-10 and IFN-γ synergize for NO production in J774 cells. (A and B) After 18 h of preincubation with 50 μg of MTSA-10 per ml, J774 cells were washed several times, rested for 2 h, and then were stimulated with 100 U of IFN-γ per ml (A) or 50 μg of *M. tuberculosis* cell lysate (MtbLys) per ml (B), in the presence or absence of 10 U of IFN-γ per ml. (C and D) J774 cells were incubated with different concentrations of MTSA-10 (C) or IFN-γ (D), with or without 10 U of IFN-γ per ml (C) or 50 μg of MTSA-10 per ml (D). Nitrite accumulation was measured in cell culture supernatants after 24 h of incubation. Control values (B) were 5.9 μM (MtbLys) and 26.2 μM (MtbLys/IFN-γ) (the nitrite level in cultures stimulated with IFN-γ alone was <1 μM). *, P of <0.01 compared to corresponding unstimulated cultures (B) or MTSA-10 (D).

LPS (9.8 ± 0.3 μM in control culture versus 3.0 ± 0.4 μM in culture with proteinase K-treated MTSA-10; $P < 0.01$; after digestion of MTSA-10, proteinase K was inactivated by boiling; a corresponding amount of boiled proteinase K was added to the control culture). Finally, both NO and TNF-α were readily induced in J774 cells by HPLC-purified MTSA-10 (data not shown), confirming that these effects were not due to the presence of contaminating proteins.

Involvement of MAPK/ERK and PTK activity in MTSA-10 effects. Next, the intracellular signaling pathways responsible for MTSA-10 effects on J774 macrophages were investigated by using the protein kinase inhibitors genistein, SB203580, and U0126, which, respectively, block the activities of protein tyrosine kinases (PTK), p38 mitogen-activated protein kinase (MAPK), and p42/p44 MAPK/ERK (through inhibition of an upstream activator, MEK). Both MTSA-10-induced TNF-α release and MTSA-10–IFN-γ-triggered NO synthesis in J774 cells were suppressed in a dose-dependent fashion by all three protein kinase inhibitors (Fig. 5A and B). For NO production, cells were pretreated with IFN-γ and then challenged with MTSA-10, to avoid interference of the kinase inhibitors with IFN-γ signals. On the other hand, MTSA-10 desensitization of macrophages for LPS-induced NO release was partly but significantly prevented by interfering with p38 MAPK but not ERK or PTK activity during MTSA-10 pretreatment (Fig. 5C). The viability of the cells was not affected by any of the inhibitors used (data not shown).

MTSA-10 effects on primary macrophages. As previously observed with the J774 cell line, flow-cytometric analysis showed that MTSA-10 can also bind to the surfaces of BMMs, although to a lesser extent (Fig. 6A). Furthermore, MTSA-10 readily synergized with IFN-γ for induction of NO release in both BMMs and peritoneal macrophage cultures (Fig. 6B and C). The effect was fairly specific for MTSA-10, since a 19-kDa fragment of *Plasmodium falciparum* merozoite surface protein 1, similarly expressed as polyhistidine-tagged protein from the pQE vector and purified by Ni-NTA chromatography, failed to affect NO production in

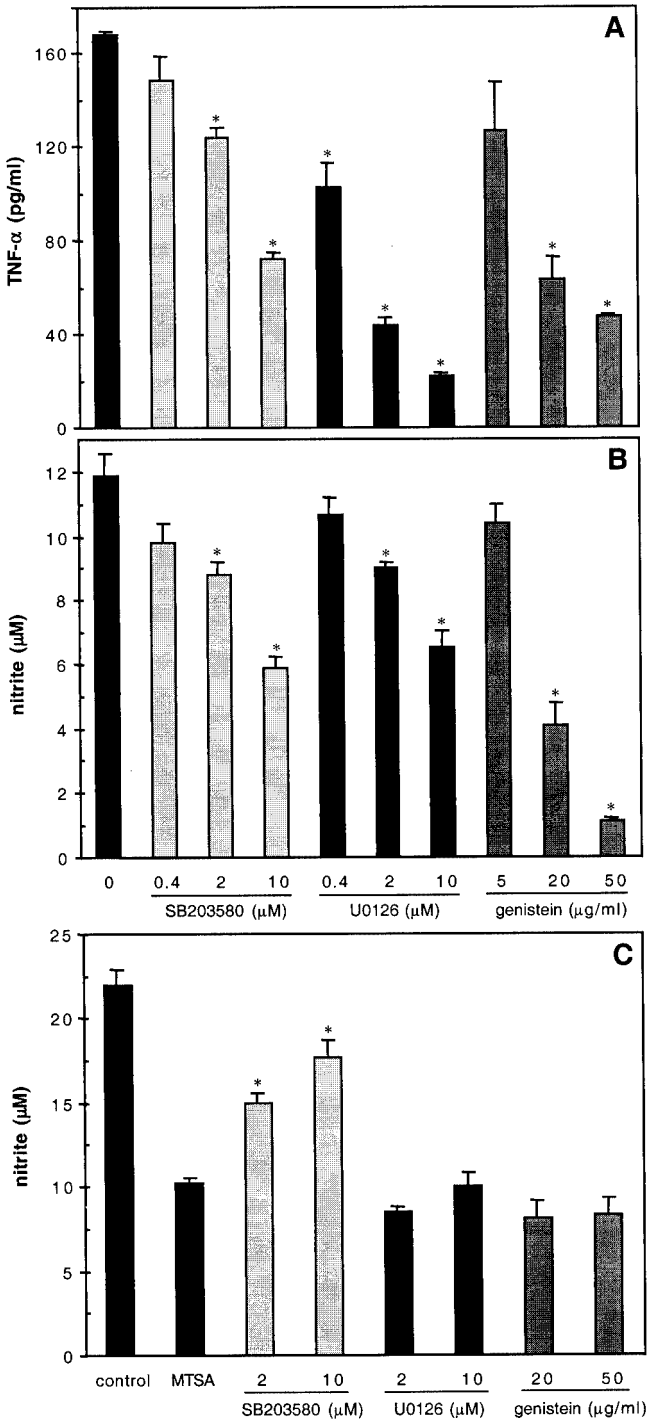

FIG. 5. Involvement of MAPK/ERK and PTK activity in MTSA-10 effects. (A) J774 cells were stimulated with 50 μg of MTSA-10 per ml in the absence or presence of different protein kinase inhibitors. (B) J774 cells were pretreated with 10 U of IFN-γ per ml for 18 h, washed, and then stimulated with 50 μg of MTSA-10 per ml, in the absence or presence of the kinase inhibitors. (C) J774 cells were incubated in medium (control) or with 50 μg of MTSA-10 per ml for 18 h, in the absence or presence of the inhibitors. After extensive washing, cells rested for 2 h and then were stimulated with 1 μg of LPS per ml. TNF-α (A) or nitrite (B and C) accumulation was measured in cell culture supernatants after 24 h of incubation *, P of <0.01 relative to cultures without any protein kinase inhibitor (A and B) or cultures pretreated with MTSA-10 alone. All inhibitors were added to cell cultures 30 min before stimulation. The cultures without the inhibitors were supplemented with 0.1% dimethyl sulfoxide, which was used as a solvent for SB203580 and U0126.

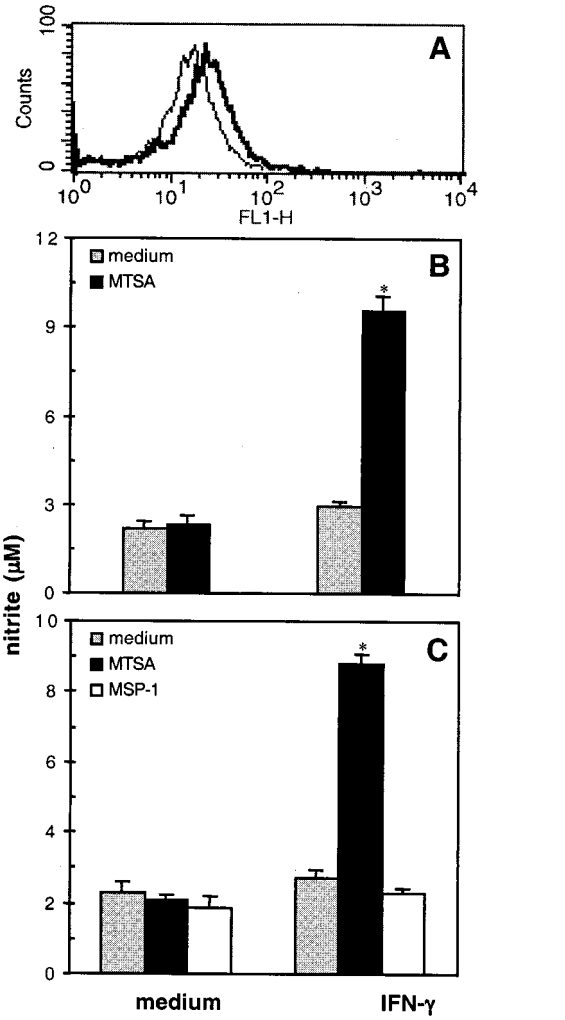

FIG. 6. Effects of MTSA-10 on primary macrophages. (A) BMMs were incubated with biotinylated MTSA-10 (50 μg/ml) and then stained with streptavidin-FITC (thick line), as described in Material and Methods. Cells stained only with streptavidin-FITC were used as a control (thin line). (B) BMMs were incubated with or without IFN-γ (10 U/ml) in the presence or absence of MTSA-10 (50 μg/ml). (C) Peritoneal macrophages were incubated with or without IFN-γ (10 U/ml), in the presence or absence of MTSA-10 (50 μg/ml) or *P. falciparum* merozoite surface protein 1 (MSP-1; 50 μg/ml). Nitrite accumulation was determined after 24 h of incubation (B and C). *, P of <0.01 relative to corresponding unstimulated cultures.

peritoneal macrophages (Fig. 6C). These data indicate that MTSA-10 might have the ability to influence the function of primary mouse macrophages.

DISCUSSION

The present study shows that MTSA-10, a secreted antigen of *M. tuberculosis*, can bind to the macrophage surface and

induce TNF-α and NO release, by itself or in synergy with IFN-γ, respectively. However, pre-exposure to MTSA-10 desensitized macrophages for subsequent production of NO but not TNF-α or the anti-inflammatory cytokine IL-10. This ability of MTSA-10 to profoundly modulate macrophage function might be relevant for the immunopathogenesis of tuberculosis.

The role of TNF-α in tuberculosis is regarded as mainly beneficial, involving the recruitment of the immune cells necessary for sealing up infectious foci inside granulomas in mice (22). It is therefore conceivable that the stimulation of macrophage TNF-α release by MTSA-10 seen in the present study might contribute to induction of host protective immunity. A similar role has been previously proposed for TNF-α induction by another secreted antigen of *M. tuberculosis*, known as 85B or the 30-kDa α antigen (4). However, studies with human monocytes and alveolar macrophages have described promotion of the intracellular replication of bacilli by TNF-α (9, 16), thus raising the possibility that TNF-α secretion induced by secreted mycobacterial antigens, including MTSA-10, may also serve as an evasion mechanism for *M. tuberculosis*. Furthermore, MTSA-10 may also participate in sustained TNF-α secretion accompanying the persistence of mycobacteria in infected macrophages and leading to self-tissue destruction during progressive disease. Indeed, it has been recently shown that administration of mycobacterial antigens to mice with prior *M. tuberculosis* infection leads to exacerbation of lung pathology via TNF-α-induced inflammation (28).

A large body of evidence, including a higher susceptibility to infection in iNOS inhibitor-treated or iNOS knockout animals (11, 25), argues in favor of the proposed involvement of iNOS-derived NO in mycobacterial clearance in mice (10). Although more controversial in humans, the role of NO in mycobacterial killing is supported by in vitro studies with *M. tuberculosis*-infected human monocytes and alveolar macrophages (21, 33, 42). In the present study, MTSA-10 desensitized murine J774 macrophages for NO release triggered by subsequent exposure to LPS or lysed *M. tuberculosis* cells. If such a mechanism operates in vivo, it might reduce the ability of MTSA-10-preexposed macrophages to carry out NO-mediated killing of intracellular bacilli upon subsequent infection. MTSA-10 pretreatment did not affect macrophage TNF-α or IL-10 synthesis, indicating that desensitization for NO release did not result from altered production of these cytokines, which are known to induce and suppress iNOS activation, respectively (43). A different pattern of cytokine synthesis observed after MTSA-10 or LPS pretreatment also suggests that distinct mechanisms were used by these two agents for reprogramming the macrophage function.

A potent macrophage activator, IFN-γ, is a hallmark of an effective immune response in tuberculosis, with its protective action being mediated mainly through induction of mycobactericidal NO synthesis in macrophages (13, 19). In the present study, IFN-γ synergized with MTSA-10 for NO induction in J774 macrophages if applied before or simultaneously with MTSA-10. However, MTSA-10 and IFN-γ seem to trigger distinct intracellular events in macrophages, since the former induced TNF-α but no NO release, while the latter caused significant NO but only marginal TNF-α production (unpublished observation). Synergistic induction of NO synthesis by IFN-γ and MTSA-10 was probably a consequence of iNOS activation, as it was sensitive to aminoguanidine, an NOS inhibitor fairly selective for its inducible isoform (27). MTSA-10 did not stimulate macrophage NO release when administered before IFN-γ, but it also failed to suppress it, in contrast to a marked inhibition of macrophage response to LPS or *M. tuberculosis* lysate as a second stimulus. Moreover, the presence of IFN-γ during restimulation with the lysed mycobacteria almost completely blocked the down-regulation of macrophage NO production imparted by preincubation with MTSA-10. One could, therefore, envisage that the effect of MTSA-10 on macrophage NO release in tuberculosis would depend on IFN-γ, in the relative absence of which the inhibitory MTSA-10 action would prevail. On the other hand, the presence of IFN-γ at the time of macrophage infection could neutralize the desensitizing effect of the prior exposure to MTSA-10. Moreover, if present before or during macrophage recognition of MTSA-10, IFN-γ might completely overcome the inhibitory action of the former, by synergizing with it for NO release. As with TNF-α, the implications of such a modulation of macrophage NO production by MTSA-10 are not straightforward, due to the complex, "double-edged sword" nature of NO involvement in the pathogenesis of tuberculosis. In addition to being a major mycobactericidal molecule, NO might exert potentially detrimental effects through suppression of the antibacterial T-cell response (29). High-level NO production is also responsible for the apoptosis of macrophages infected with *M. tuberculosis* (34). Because it is secreted, MTSA-10 can contribute to iNOS induction and consequent apoptosis in uninfected macrophages as well, thus reducing the protective capacity of the host. Indeed, we have observed NO-dependent reduction of cellular respiration, assessed by the MTT assay, in macrophage cultures treated with a combination of MTSA-10 and IFN-γ (unpublished observation).

The activation of PTK as well as the serine/threonine kinases p38 MAPK and ERK (p42/44 MAPK) has been implicated in the induction of TNF-α and NO synthesis in macrophages stimulated with various microbial products (15, 17, 30, 35, 38). Our data indicate that these signaling pathways are also responsible for MTSA-10-triggered release of TNF-α and NO in J774 macrophages. Although the cells were preincubated with IFN-γ, which was washed away prior to NO induction with MTSA-10, we could not completely exclude the possibility that IFN-γ-dependent intracellular events contributing to NO release were also interrupted by the kinase inhibitors. However, the dependence of MTSA-10-induced TNF-α release on p38 MAPK, ERK, and PTK activity suggests that the same pathways may also control MTSA-10 induction of macrophage iNOS. This finding also indicates that MTSA-10–IFN-γ-induced NO release might be partly mediated through the autocrine-paracrine action of endogenous TNF-α, as previously reported for *M. tuberculosis*-infected macrophages (34). Interestingly, only p38 MAPK activity, not ERK or PTK activity, seems to be involved in MTSA-10-mediated desensitization of macrophages for LPS-stimulated NO production. The activation of p38 MAPK has been recently found to be responsible for the down-regulation of LPS-induced TNF-α release in rats previously exposed to sublethal hemorrhage (26). However, since MTSA-10 in the present study did not impair macrophage ability to secrete TNF-α in response to LPS, it appears

that some other mechanisms besides p38 MAPK activation are involved in macrophage desensitization for TNF-α production.

The regulation of macrophage TNF-α and NO production in tuberculosis appears to be very complex, due to the ability of various mycobacterial cell wall components to stimulate the release of these inflammatory mediators (6, 32, 40). However, the secreted mycobacterial proteins, such as MTSA-10, might have the advantage in that respect, since their soluble nature might enable them to affect a wider population of macrophages. The presence of anti-MTSA-10 antibodies in the sera of tuberculosis patients (8, 14) indicates that MTSA-10 from infected macrophages or unphagocytosed bacteria might indeed gain access to the extracellular compartment, thus acquiring the opportunity to influence macrophage function in tuberculosis. Alternatively, macrophages could recognize MTSA-10 expressed in the cell wall of *M. tuberculosis*, as previously proposed for 85B, another secreted *M. tuberculosis* antigen (4). Finally, direct macrophage activation for TNF-α and NO production seems likely to contribute to development of delayed-type hypersensitivity reaction after administration of MTSA-10 to *M. tuberculosis*-infected animals (12, 41). The putative modulation of macrophage proinflammatory activity by MTSA-10 might be of great importance for designing MTSA-10-based vaccines or diagnostic tools for tuberculosis. It remains for future studies, however, to explore whether the presented findings apply to human macrophages as well as to in vivo situations.

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